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Complete Regression of Established Murine Hepatocellular Carcinoma by In Vivo Tumor Necrosis Factor α Gene Transfer

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See editorial on page 656.

Background & Aims: **Although tumor necrosis factor (TNF)-α possesses a potent antitumor activity, systemic administration of TNF-α causes severe side effects. To circumvent this, the efficacy of tumor cell–targeted TNF-α gene therapy was investigated.** ***Methods:*** **Murine hepatocellular carcinoma (HCC) cells were infected with MNSM-Alb e/p-TNF-α retroviruses carrying the murine TNF-α gene under the transcriptional control of the murine albumin gene promoter, and antitumor effects induced by TNF-α gene transfer were examined in vitro and in vivo.** ***Results:*** **Although MNSM-Alb e/p-TNF-α retrovirally infected HCC cells showed the same in vitro cell growth as parental HCC cells, they lost their tumorigenicity when implanted in syngeneic mice and induced tumor immunity against parental HCCs. The retrovirally infected HCC cells also significantly inhibited the tumorigenicity of previously implanted parental HCCs. Furthermore, intratumoral administration of MNSM-Alb e/p-TNF-α retroviruses showed the antitumor effect against established HCCs, resulting in significantly prolonged survival periods. Most importantly, intratumoral implantation of MNSM-Alb e/p-TNF-α retroviral–producing cells completely abrogated established HCCs in mice.** ***Conclusions:*** **These results indicate the potential efficacy of transferring the TNF-α gene via retroviral vectors directly into tumors for gene therapy against HCCs.**

Systemic administration of tumor necrosis factor (TNF)-α has been proven to induce the regression of a number of established experimental murine tumors.[1] However, it has little therapeutic effect when applied clinically for the treatment of cancers, because humans can tolerate only 2% of the dose required for tumor regression in mice.[2,3] Therefore, application of therapeutically effective doses of TNF-α is often accompanied by severe side effects. This problem may be circumvented by tumor cell–targeted cytokine gene therapy.[4] For instance, it was shown that intratumoral administration of TNF-α induced tumor necrosis in animal models.[5,6] Furthermore, tumor cells genetically engineered to produce TNF-α in vitro were shown to provide a locally enhanced TNF-α concentration and lose their tumorigenicity in mice.[7–9] It was also reported that the amounts of TNF-α released by genetically engineered tumor cells correlated with the intensity of the tumor rejection.[2,8] Therefore, if high intratumoral concentrations of TNF-α can be achieved, efficient antitumor effects against established tumors may be induced without systemic toxicity.

We have been working on gene therapy against hepatocellular carcinoma (HCC) using recombinant retroviruses in animal models, because HCC is one of the most common malignancies in Asia.[10,11] There is still no satisfactory treatment that significantly improves the overall survival rate of patients with HCCs.[12,13] Most patients with HCCs have underlying liver cirrhosis. Therefore, it is not practical in the majority of patients with HCCs to collect surgically the target HCC cells for genetic modification, because of their limited hepatic reserve. In vivo gene transfer methods are definitely more applicable for the treatment of patients with HCCs. Retroviruses provide a potential means of selectively infecting and achieving integration into the genome of dividing cells.[14] Therefore, retroviruses are an attractive vector for cancer cell–specific gene transfer, because most normal cells surrounding cancers are in a quiescent, nonreceptive stage of cell growth. There are, however, noncancerous normal dividing cells, such as bone marrow cells and intestinal epithelial cells. Therefore, tumor cell–specific gene delivery of therapeutic genes must be developed for gene therapy against cancers using retroviral-mediated in vivo

Abbreviations used in this paper: CFU, colony-forming units; SDS, sodium dodecyl sulfate; SV 40, simian virus 40; TNF, tumor necrosis factor.

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...transfer. We have already shown retroviral-mediated HCC- and glioma-specific gene delivery both in vitro and in vivo by using the albumin gene promoter[19] and myelin basic protein gene promoter[20] as internal promoters in retroviral vectors, respectively. Another important factor to achieve an antitumor effect is to ensure a strong expression of transferred therapeutic genes in vivo. We have shown that the albumin gene promoter can induce stronger expression of exogenous genes in HCC cells in vitro than the simian virus 40 (SV 40) early region promoter, which is widely used to induce strong expression of exogenous genes in various tumor cells.[21]

In the present study, we examined whether murine TNF-α gene expression directed by the murine albumin enhancer/promoter or SV 40 early region promoter can abrogate the tumorigenicity of murine HCC cells in vivo. Furthermore, we investigated whether the retroviral-mediated in vivo transduction of the TNF-α gene can induce antitumor effects on established HCCs in animals.

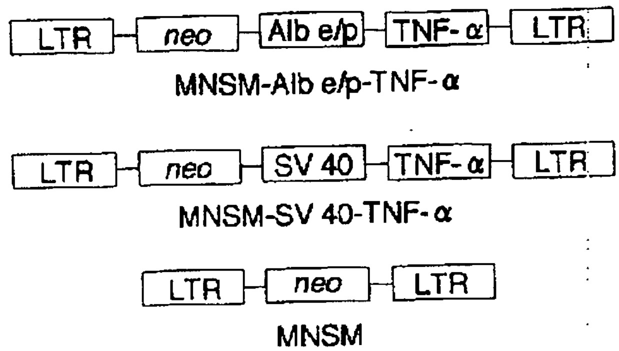

Figure 1. Structure of the recombinant retroviral vectors. The MNSM-Alb e/p-TNF-α retroviral vector contains the neomycin phosphotransferase (*neo*) gene, which confers G418 resistance on transfected cells, the murine albumin enhancer and promoter (Alb e/p) fragment, as an internal promoter, and the murine TNF-α gene within two Moloney murine leukemia virus long-terminal repeats (LTRs). The MNSM-SV 40-TNF-α retroviral vector contains the SV 40 early region promoter as an internal promoter in place of the murine Alb e/p fragment of the MNSM-Alb e/p-TNF-α vector. The MNSM retroviral vector contains the only *neo* gene without the TNF-α gene.

Materials and Methods

Cell Lines and Cell Culture

The murine HCC cell line MM45T.Li, murine embryo fibroblast line NIH3T3,[22] and amphotropic retrovirus packaging cell line PA317[23] were purchased from American Type Culture Collection (Rockville, MD). The cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 U/mL ampicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2 in air.

Structure of the Retroviral Vectors

All plasmids were constructed by standard recombinant DNA techniques.[24,25] Construction of the MNSM-Alb e/p-TNF-α and MNSM-SV 40-TNF-α retroviral plasmids has been described previously,[21] and the diagrams are shown in Figure 1. The MNSM-Alb e/p-TNF-α retroviral vector contains the neomycin phosphotransferase gene,[26] which confers G418 resistance on transfected cells; the murine albumin enhancer and promoter element,[27] as an internal promoter; and the murine TNF-α gene[28] within two Moloney murine leukemia virus long-terminal repeats.[29] The MNSM-SV 40-TNF-α retroviral vector contains the SV 40 early region promoter as an internal promoter in place of the murine albumin enhancer and promoter element of the MNSM-Alb e/p-TNF-α vector. The MNSM retroviral vector[30] containing the only neomycin phosphotransferase gene without the TNF-α gene was used as a control retroviral vector.

Recombinant Retroviral Production and Gene Transfer

Production of replication-defective amphotropic retroviral vectors was accomplished by the calcium phosphate precipitation procedure as described previously.[31] Briefly, PA317 amphotropic retroviral packaging cells were plated at 5×10^5 cells per 60-cm dish. On the following day, DNA-$CaCl_2$ solution was prepared by mixing 25 µL of 2.0 mol/L $CaCl_2$, 10 µg of plasmid DNA in 10 mmol/L Tris-HCl at pH 7.5, and water to make 200 µL total. Precipitation buffer was freshly prepared by mixing 100 µL of 500 mmol/L HEPES-NaOH at pH 7.1, 125 µL of 2.0 mol/L NaCl, 10 µL of 150 mmol/L Na_2HPO_4–NaH_2PO_4 at pH 7.0, and water to make 1 mL total. DNA-$CaCl_2$ solution (200 µL) was added dropwise with constant agitation to 200 µL of precipitation buffer. After 30 minutes at room temperature, the resultant fine precipitates were added to a dish of PA317 cells. Two days later, the medium was aspirated and fresh medium containing 1 mg/mL G418 (Gibco, Grand Island, NY) was added. Independent G418-resistant clones were isolated and expanded as retroviral-producing cells. The high-titer retroviral-producing clones selected for subsequent experiments had a titer of approximately 1×10^7 colony-forming units (CFU)/mL as assayed by G418 selection of infected NIH3T3 cells. The culture supernatant of the high-titer retroviral-producing clone was collected, passed through a 0.45-µm-pore filter (Millipore Corp., Bedford, MA), and stored at −70°C. It served as a source for infectious recombinant retroviruses.

Freshly prepared murine HCC cells were infected with the recombinant retroviruses at 37°C, 5% CO_2 for 4–6 hours in the medium containing 8 µg/mL polybrene (Sigma Chemical Co., St. Louis, MO). The medium was replaced with a fresh virus-free one, and the cells were incubated for 2 days. Infected cells were then selected by addition of G418 to a final concentration of 1 mg/mL, and the bulk of G418-resistant cells were expanded for subsequent experiments.

TNF-α Production

To determine whether the retroviral-infected cells produce biologically active TNF-α, a sandwich enzyme-linked immunosorbent assay technique was used. Rat anti-murine TNF-α monoclonal antibodies MPG-XT3 and MP6-XT 22 (Gibco), which recognize different epitopes on murine TNF-α, were used for quantitative measurement of murine TNF-α. Briefly, microtiter plates were coated with the MPG-XT3 antibody. After treatment with a blocking solution, the supernatants of retroviral-infected cells and recombinant murine TNF-α (Gibco) in serial dilution were added to the plates. The peroxidase-conjugated MP6-XT22 antibody was then added to the plates for sandwiching murine TNF-α. After washing, the revealing solution containing tetramethylbenzidine (Boehringer Mannheim, Germany) was added and incubated. The intensity of the resulting color was determined photometrically by an automated microplate reader (Bio-Rad, Hercules, CA). Absorbance was proportional to recombinant murine TNF-α concentration. A standard curve was plotted and TNF-α concentration in samples was determined by interpolation from the standard curve.

Tumorigenicity and Tumor Immunity

All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals.

Murine HCC cells modified with retroviral-mediated gene transfer were suspended in phosphate-buffered saline (PBS) at a concentration of 5×10^7 cells/mL, and 100-μL inoculum volumes were injected subcutaneously into the right flank regions of syngeneic BALB/c mice. Control mice were injected subcutaneously with the same number of unmodified parental HCC cells suspended in the same volume of PBS. After the injection, the mice were ear tagged at random and observed for subcutaneous tumor formation. Tumor size was determined by measuring the largest diameter. Mice bearing a tumor larger than 3 mm in the largest diameter were considered positive.

For tumor immunity assessment, mice that did not develop subcutaneous tumors were rechallenged with 5×10^6 parental HCC cells (100-μL inoculum volumes), a tumorigenic dose in 100% of animals, in the vicinity of the previous inoculation. They were observed for tumor development as described previously.

Antitumor Effect Induced by Modified Cells

Five million parental HCC cells suspended in 100 μL of PBS were injected subcutaneously into the flank regions of syngeneic BALB/c mice. Three days after the inoculation, the same number of HCC cells (100-μL inoculum volumes) infected with the recombinant retroviruses was injected into the same vicinity. Tumor growth was estimated every 2 or 3 days as described previously.

Antitumor Eff ct Caused by In Vivo Transduction of the TNF-α Gen

To examine the antitumor effect induced by in vivo retroviral administration, 5×10^6 parental HCC cells were injected subcutaneously into the syngeneic mice. When subcutaneous tumors reached ~10 mm in the largest diameter, animals in the treatment groups were injected intratumorally or peritoneally with 2×10^6 CFU of retroviruses contained in 200 μL of the complete medium with 8 μg/mL polybrene for 5 consecutive days. Control mice received intratumoral injection of the same volume of the complete medium with 8 μg/mL polybrene. Each group consisted of 10 animals. The tumor size and survival rate of the animals were monitored every 2 or 3 days.

To examine the antitumor effect induced by implantation of the retroviral-producing cells, the mice bearing an established HCC were implanted intratumorally with 2×10^6 retroviral-producing cells suspended in 200 μL of the conditioned medium with 16 μg/mL polybrene for 5 consecutive days. Each group consisted of five animals. The tumor size and survival rate of animals were monitored every 2 or 3 days.

Northern Blot Assay

The mice bearing an established parental HCC were implanted intratumorally with 2×10^6 retroviral-producing cells in 200-μL inoculum volumes containing 16 μg/mL polybrene for 5 consecutive days. Seven days after the first implantation, the tumors were excised and homogenized in a homogenate buffer containing 50 mmol/L NaCl, 50 mmol/L Tris-HCl at pH 7.5, 5 mmol/L ethylenediaminetetraacetic acid at pH 8.0, 0.5% sodium dodecyl sulfate (SDS), and 200 mg/mL proteinase K (Sigma). Total cellular RNA from the tumor was extracted by the guanidine-isothiocyanate technique.[24] Thirty micrograms of RNA samples was electrophoresed in a 1% agarose/2.2 mol/L formaldehyde gel and transferred to a nylon membrane. The membrane was cross-linked with UV light and hybridized with the murine TNF-α complementary DNA (cDNA) probe at 68°C. The probe was radiolabeled with ^{32}P-labeled deoxycytidine triphosphate by the random priming method (Promega Biotec, Madison, WI). The membrane was washed twice for 15 minutes at room temperature with 2× standard saline citrate/0.1% SDS and once at 60°C for 30 minutes with 0.1× standard saline citrate/0.1% SDS. The membrane was then imaged by contact with an x-ray film. After removal of the probe, the membrane was rehybridized with the radiolabeled chicken β-actin cDNA[32] to ensure lane-lane equivalency of RNA loading and transfer. The band densities were measured using a Macintosh computer (Quadra 650; Macintosh, Cupertino, CA) with the public domain NIH Image program written by Wayne Rasband at the National Institutes of Health (Bethesda, MD).

Histological Evaluation and Immunohistochemical Staining

Subcutaneous parental HCCs were excised from the mice 14 and 21 days after the first implantation of retroviral-producing cells. The tumors were fixed in 10% buffered formalin, embedded in paraffin, and stained with hemalaun for routine histology. Indirect immunoenzymatic staining of 6-μm

...zen sections was performed by using the following rat anti-mouse monoclonal antibodies: L3T4 (ATCC, TIB 207), Lyt2 (ATCC, TIB 105), IL-2R (ATCC, CRL 1698), and Mac-1 (ATCC, TIB 128). The slides were then incubated sequentially with biotin-conjugated goat anti-mouse immunoglobulins (Vector Laboratories Inc., Burlingame, CA). Sections were then incubated with 0.02% hydrogen peroxide (Sigma) and 0.1% diaminobenzidine tetra hydrochloride (Vector), washed in tap water, and counterstained by hemalaun.

Statistics

Standard descriptive statistics, the Student's t test and χ^2 test, were used. A P value of <0.05 was considered significant.

Results

Generation Time of Retroviral-Infected HCC Cells

First, the retroviral-mediated TNF-α gene transfer into murine HCC cells was examined for any cytotoxic effects on cell proliferation. HCC cells infected with MNSM, MNSM-SV 40-TNF-α, or MNSM-Alb e/p-TNF-α retroviruses, as well as their parental cells, were seeded at a density of 1×10^3 cells/cm^2 in the complete medium, and the number of viable cells was determined after trypan blue staining. All determinations were carried out in three separate experiments. All the cell lines infected with the retroviruses showed the same growth ratios as uninfected parental cells (data not shown), and the generation time of all clones was approximately 19 hours.

TNF-α Production by Retroviral-Infected HCC Cells

Production of TNF-α by retroviral-infected HCC cells was estimated by determining biologically active TNF-α concentrations of the culture media. As shown in Table 1, parental and MNSM retroviral–infected HCC cells did not produce detectable levels of TNF-α. Conversely, the cells infected with MNSM-SV 40-TNF-α and MNSM-Alb e/p-TNF-α retroviruses did secrete 1406 and 6685 pg/10^6 cells per 24 hours of TNF-α on the average into the culture media, respectively. The cells infected with MNSM-Alb e/p-TNF-α retroviruses produced significantly higher levels of TNF-α than those infected with MNSM-SV 40-TNF-α retroviruses.

Tumorigenicity of Retroviral-Infected HCC Cells and Tumor Immunity Against Parental HCCs

To examine the tumorigenicity of HCC cells infected with retroviruses, syngeneic BALB/c mice were implanted subcutaneously with 5×10^6 HCC cells infected with the MNSM, MNSM-SV 40-TNF-α, or MNSM-Alb e/p-TNF-α retrovirus. As shown in Table 2, all the mice inoculated with parental HCC cells, and 8 of the 10 mice inoculated with MNSM retroviral–infected HCC cells developed subcutaneous tumors within 20 days, whereas only 1 and none of the 10 mice inoculated with MNSM-SV 40-TNF-α and MNSM-Alb e/p-TNF-α retroviral–infected HCC cells showed tumor formation, respectively. To learn whether the inoculation of TNF-α gene–transduced HCC cells resulted in tumor immunity against parental HCCs, mice that did not develop subcutaneous tumors were again implanted with 5×10^6 parental HCC cells in the vicinity of the previous injection. The animals previously inoculated with MNSM-SV 40-TNF-α and MNSM-Alb e/p-TNF-α retroviral–infected cells rejected the subsequent challenge of wild-type HCC, with tumor incidence of 33% and 10%, respectively.

Inhibition of Tumorigenicity of Parental HCC Cells Induced by Retroviral-Infected HCC Cells

Parental HCC cells (5×10^6 cells/100 μL PBS) were implanted subcutaneously into the flank region of

Table 1. TNF-α Content of Supernatants From Parental and Modified HCC Cells

Retrovirally modified cells	TNF-α content (*pg/10^6 cells/24 h*)
None (parental)	BT
MNSM	BT
MNSM-SV 40-TNF-α	1406 ± 624
MNSM-Alb e/p-TNF-α	6685 ± 1302[a]

NOTE. Results are expressed as means ± SD for five separate experiments.
BT, below threshold.
[a]Significantly different from values of MNSM-SV 40-TNF-α at $P <$ 0.001 by Student's t test.

Table 2. Tumorigenicity of Modified HCC Cells and Tumor Immunity Against Parental HCCs

Retrovirally modified cells	Tumor incidence by cell implantation (%)	Tumor incidence by rechallenge with parental cells (%)
None (parental)	10/10 (100)	ND
MNSM	8/10 (80)	2/2 (100)
MNSM-SV 40-TNF-α	1/10 (10)[a]	3/9 (33)
MNSM-Alb e/p-TNF-α	0/10 (0)[a]	1/10 (10)

ND, not done.
[a]Significantly different from the result of MNSM retrovirally modified cells at $P < 0.01$ by χ^2 test.

Table 3. Inhibition of Tumorigenicity of Parental HCC

Retrovirally modified cells	Tumor incidence of previously implanted parental cells (%)
None (parental)	10/10 (100)
MNSM	10/10 (100)
MNSM-SV 40-TNF-α	5/10 (50)[a]
MNSM-Alb e/p-TNF-α	1/10 (10)[b]

[a,b]Significantly different from the result of MNSM retrovirally modified cells at $P < 0.05$ and $P < 0.01$, respectively (χ^2 test).

a BALB/c mouse, and 3 days later the same number of the retroviral-infected and -uninfected counterparts suspended in 100 μL of PBS were injected into the vicinity of the previous injection. As shown in Table 3, the injection of parental and MNSM retroviral–infected HCC cells did not inhibit the tumorigenicity of the previously implanted parental HCC cells. However, the injection of MNSM-SV 40-TNF-α and MNSM-Alb e/p-TNF-α retroviral–infected HCC cells did significantly inhibit the tumorigenicity of the previously implanted parental HCC cells, with the incidence of detectable tumor development of 50% and 10%, respectively. The inhibitory effect on tumorigenicity of previously implanted parental HCC cells induced by MNSM-Alb e/p-TNF-α retroviral–infected HCC cells was stronger than that induced by MNSM-SV 40-TNF-α retroviral–infected ones, although the difference was not statistically significant.

Anti-HCC Effect Induced by In Vivo Retroviral Administration

Syngeneic BALB/c mice were implanted subcutaneously with 5×10^6 parental HCC cells. When subcutaneous HCCs reached ~10 mm in diameter, the animals received intratumoral or intraperitoneal administrations of 2×10^6 CFU of retroviruses in 200 μL of the conditioned medium with 8 μg/mL polybrene for 5 consecutive days. As shown in Figure 2, the mice that received MNSM retroviruses developed rapidly growing tumors, whereas those that received MNSM-SV 40-TNF-α or MNSM-Alb e/p-TNF-α retroviruses showed the significantly suppressed tumor growth. The diameters of the mice receiving intratumoral administration of MNSM-SV 40-TNF-α and MNSM-Alb e/p-TNF-α retroviruses were significantly smaller than those of the mice receiving intratumoral administration of MNSM retroviruses from day 7 and day 5 after the first administration, respectively. Furthermore, this antitumor effect was exerted significantly more strongly by MNSM-Alb e/p-TNF-α retroviruses than by MNSM-SV 40-TNF-α retroviruses from day 12. Conversely, the mice that received intraperitoneal administration of MNSM-Alb e/p-TNF-α retroviruses developed rapidly growing tumors, not significantly different from those that received intratumoral administration of MNSM retroviruses (data not shown). Figure 3 shows the survival rate of the mice that received the intratumoral administration of the various retroviruses. All the mice that received control MNSM retroviruses died within 14 days after the initiation of the retroviral administration, and all the mice that received MNSM-SV 40-TNF-α retroviruses also died within 35 days. On the other hand, the survival rate of the mice administered MNSM-Alb e/p-TNF-α retroviruses was significantly higher compared with that of the mice administered MNSM-SV 40-TNF-α retroviruses, and 80% of the mice were still alive even 42 days after the administration.

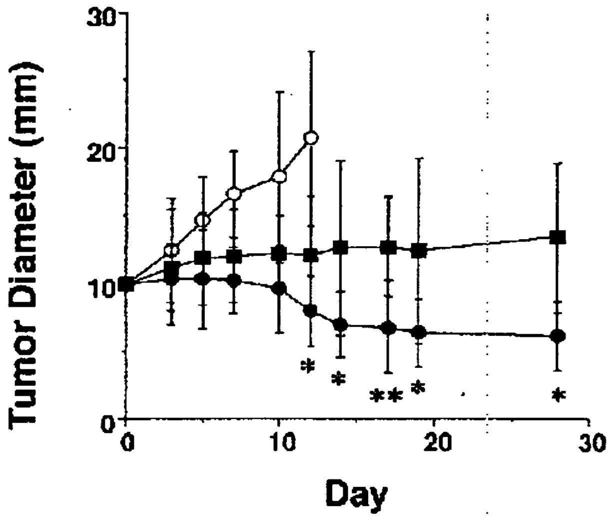

Figure 2. Anti-HCC effect induced by in vivo retroviral administration. Syngeneic mice were implanted subcutaneously with 5×10^6 parental HCC cells. When the mice developed a tumor with a diameter of ~10 mm, they were injected intratumorally with 2×10^6 CFU of MNSM (○, n = 10), MNSM-SV 40-TNF-α (■, n = 10), or MNSM-Alb e/p-TNF-α (●, n = 10) retroviruses for 5 consecutive days. Tumor diameter was measured every 2 or 3 days after the first retroviral injection. Each data point represents the mean ± SD. Tumor diameters of the mice injected with MNSM-SV 40-TNF-α and MNSM-Alb e/p-TNF-α retroviruses were significantly smaller than those of the mice injected with MNSM retroviruses from day 7 and day 5, respectively. Tumor diameters of the mice injected with MNSM-Alb e/p-TNF-α retroviruses also were significantly smaller than those of the mice injected with MNSM-SV 40-TNF-α retroviruses. * $0.01 < p < 0.05$, ** $P < 0.01$ by Student's *t* test.

Anti-HCC Effect Induced by Implantation of Retroviral-Producing Cells

The mice bearing an established parental HCC were then treated with the intratumoral implantation

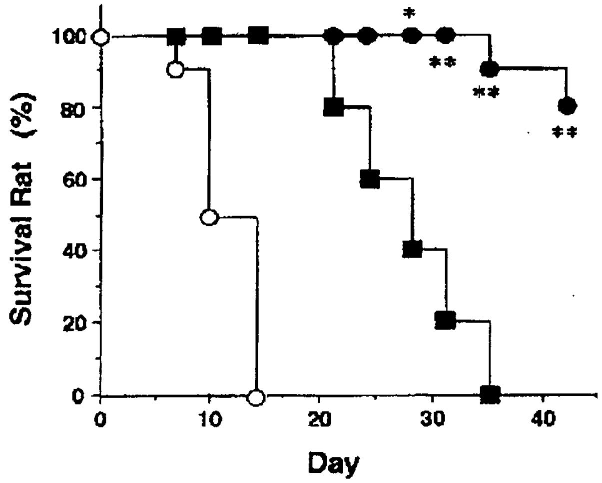

Figure 3. Survival rates of mice that received intratumoral retroviral administration. The mice bearing an established subcutaneous HCC with a diameter of ~10 mm were injected intratumorally with MNSM (○, n = 10), MNSM-SV 40-TNF-α (■, n = 10), or MNSM-Alb e/p-TNF-α (●, n = 10) retroviruses for 5 consecutive days. The survival rate of the mice injected with MNSM-Alb e/p-TNF-α retroviruses was significantly different compared with that of the mice injected with MNSM-SV 40-TNF-α retroviruses. * $0.01 < P < 0.05$, ** $P < 0.01$ by χ^2 test.

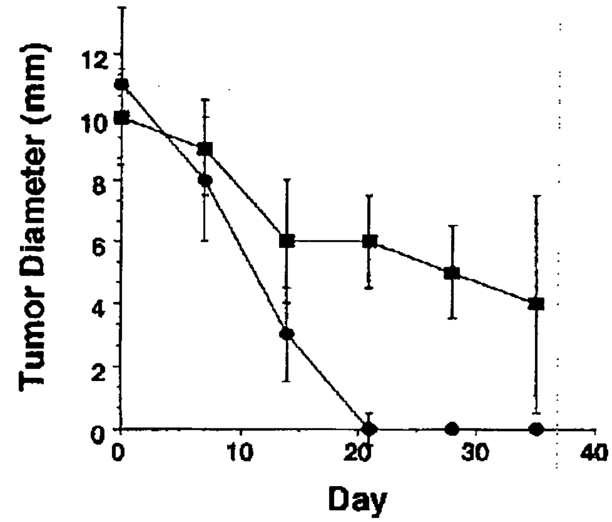

Figure 4. Anti-HCC effect induced by implantation of retroviral-producing cells. The mice bearing an established subcutaneous HCC with a diameter of ~10 mm were implanted intratumorally with 2×10^6 MNSM-SV 40-TNF-α (■, n = 5) or MNSM-Alb e/p-TNF-α (●, n = 5) retroviral-producing cells for 5 consecutive days. Tumor diameters were measured every 2 or 3 days after the first implantation. Each data point represents the mean ± SD. Reduction rates of tumor diameters induced by either retroviral-producing cells are statistically significant from day 14.

of retroviral-producing cells instead of the intratumoral retroviral administration. When subcutaneous HCCs reached ~10 mm in diameter, the animals were implanted intratumorally with the retroviral-producing cells. As shown in Figure 4, tumor size of the mice implanted with MNSM-SV 40-TNF-α retroviral–producing cells did reduce significantly, and it remained significantly smaller even 35 days after the implantation. Furthermore, all of the 5 mice that received intratumoral implantation of MNSM-Alb e/p-TNF-α retroviral–producing cells abrogated the established parental HCCs completely by 28 days after the implantation. No obvious signs of treatment-related side effects, such as body weight loss, were observed. Although 2 of the 5 mice experienced tumor recurrence 77 and 84 days after the implantation, the remaining 3 mice did not have tumors throughout the 6-month observation period.

Intratumoral Expression of the TNF-α Gene Induced by Implantation of Retroviral-Producing Cells

Seven days after the implantation of the retroviral-producing cells into established parental HCCs, the animals were killed and the tumors were excised. Total cellular RNA was extracted from the tumors, and the expression of the TNF-α gene was examined using the murine TNF-α cDNA as a probe. As shown in Figure

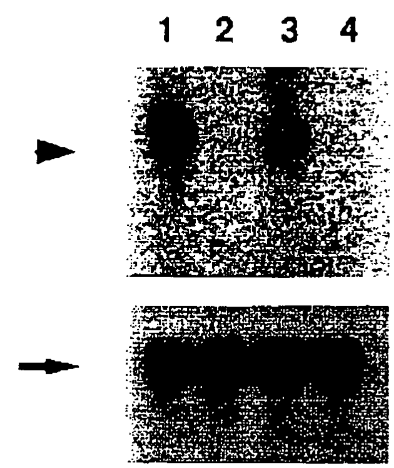

Figure 5. Intratumoral expression of the TNF-α gene induced by implantation of retroviral-producing cells. The mice bearing an established subcutaneous HCC were implanted intratumorally with retroviral-producing cells for 5 consecutive days. Seven days after the first implantation, tumors were excised and total cellular RNA was extracted. Equivalent amounts (30 μg) of RNA extracted from tumors implanted with MNSM-Alb e/p-TNF-α (*lane 1*), MNSM (*lane 2*), MNSM-SV 40-TNF-α (*lane 3*) retroviral–producing cells or PA317 retrovirus packaging cells (*lane 4*) were separated by gel electrophoresis. The RNA was blotted and hybridized to a ^{32}P-radiolabeled murine TNF-α–specific probe and then a chicken β-actin probe. The *arrowhead* and *arrow* indicate the murine TNF-α–specific and chicken β-actin–specific transcripts, respectively.

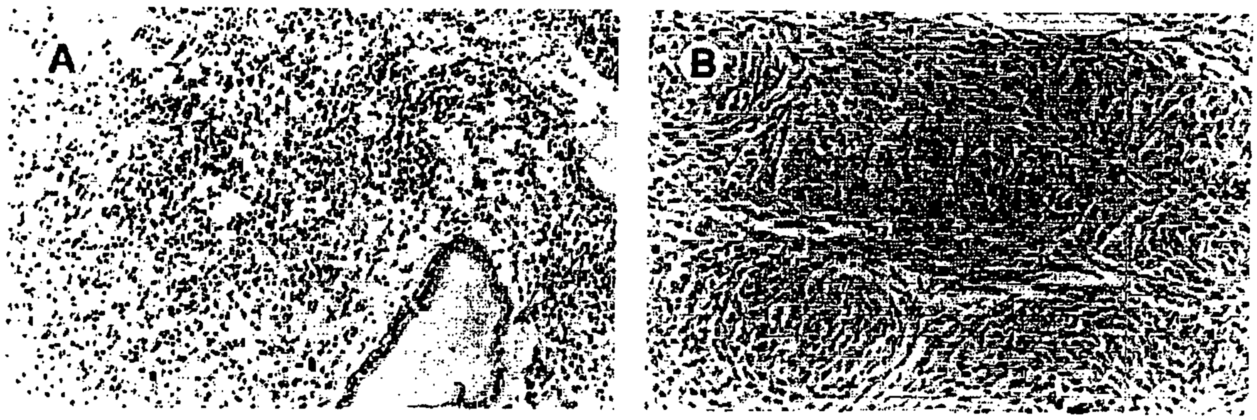

Figure 6. Histological analysis of the tumor implanted with MNSM-Alb e/p-TNF-α retroviral–producing cells. The mice bearing an established subcutaneous HCC were implanted intratumorally with MNSM-Alb e/p-TNF-α retroviral–producing cells for 5 consecutive days. (*A*) Fourteen days after the first implantation, massive necrosis, hemorrhage, and many infiltrating cells were visible in the tumor that received implantation of MNSM-Alb e/p-TNF-α retroviral–producing cells. (*B*) A much smaller number of infiltrating cells without apparent necrosis or hemorrage were seen in the tumor that received the same volume of PBS (original magnification 60×).

5, TNF-α gene expression was undetectable in the tumors that received PA317 retrovirus packaging cells or MNSM retroviral–producing cells. Conversely, expression of the TNF-α gene was readily detected in the tumors that received MNSM-SV 40-TNF-α, or MNSM-Alb e/p-TNF-α retroviral–producing cells. Densitometric scanning revealed that the expression of the TNF-α gene was approximately 1.6-fold stronger in the tumors that received MNSM-Alb e/p-TNF-α retroviral–producing cells compared with those receiving MNSM-SV 40-TNF-α retroviral–producing cells.

Histological Analysis of Tumors Implanted With Retroviral-Producing Cells

Pathological examination of the tumors that received MNSM-Alb e/p-TNF-α retroviral–producing cells was performed at different time points after the implantation. Fourteen days after the implantation of the retroviral-producing cells, massive necrosis, hemorrhage, and infiltrating cells were visible in the tumor (Figure 6*A*). Twenty-one days later, infiltrating cells and fibroblasts but not tumor cells were visible in the tumor, and the residual tumors were actually fibrotic scars devoid of tumor cells (data not shown). Conversely, control tumors that received the same volume of PBS instead of the retroviral-producing cells contained a much smaller number of accompanying infiltrating cells and no apparent necrosis or hemorrhage (Figure 6*B*). To identify the infiltrating cells at the tumor site, immunohistochemical analysis was performed 14 days after the intratumoral implantation of MNSM-Alb e/p-TNF-α retroviral–producing cells. The reactive cells were positive to Mac-1 (Figure 7*A*), Lyt2 (Figure 7*B*), and IL-2R (Figure 7*C*) staining, but negative to L3T4 staining (data not shown).

Discussion

TNF-α is a cytokine that possesses a wide variety of biological activities including potent antitumor activity.[1,33–39] However, studies of TNF-α–mediated tumor regression in mice have been hampered by the need to systemically administer toxic doses to obtain a curative response.[1,6,40] The problem of dose-limiting toxicity of TNF-α has been particularly evident in human trials, because the maximal tolerated dose of TNF-α in humans has been shown to be 40-fold less on a per kilogram basis than the doses that were required to induce a significant antitumor effect in mice.[1,2] The severe side effects, such as headache, fever, tachycardia, rigors, and vomiting, observed in clinical trials in human cancer patients using TNF-α[3,41] may be avoidable if high local concentrations of TNF-α can be achieved solely in tumor sites. Blankenstein et al.[7] and Asher et al.[8] showed murine tumor cell lines engineered to produce TNF-α by retroviral-mediated gene transfer lost their tumorigenicity in syngeneic mice. Recently, Han et al.[42] also showed that a human lung cancer cell line modified to secrete human TNF-α lost its tumorigenicity in athymic mice.

In the present study, the murine TNF-α gene was transduced into a murine HCC cell line by retroviral vectors. HCC cells infected with either MNSM-Alb e/p-TNF-α or MNSM-SV 40-TNF-α retroviruses secreted biologically active TNF-α, whereas those infected with control MNSM retroviruses did not produce any detectable levels of TNF-α. TNF-α gene–transduced HCC

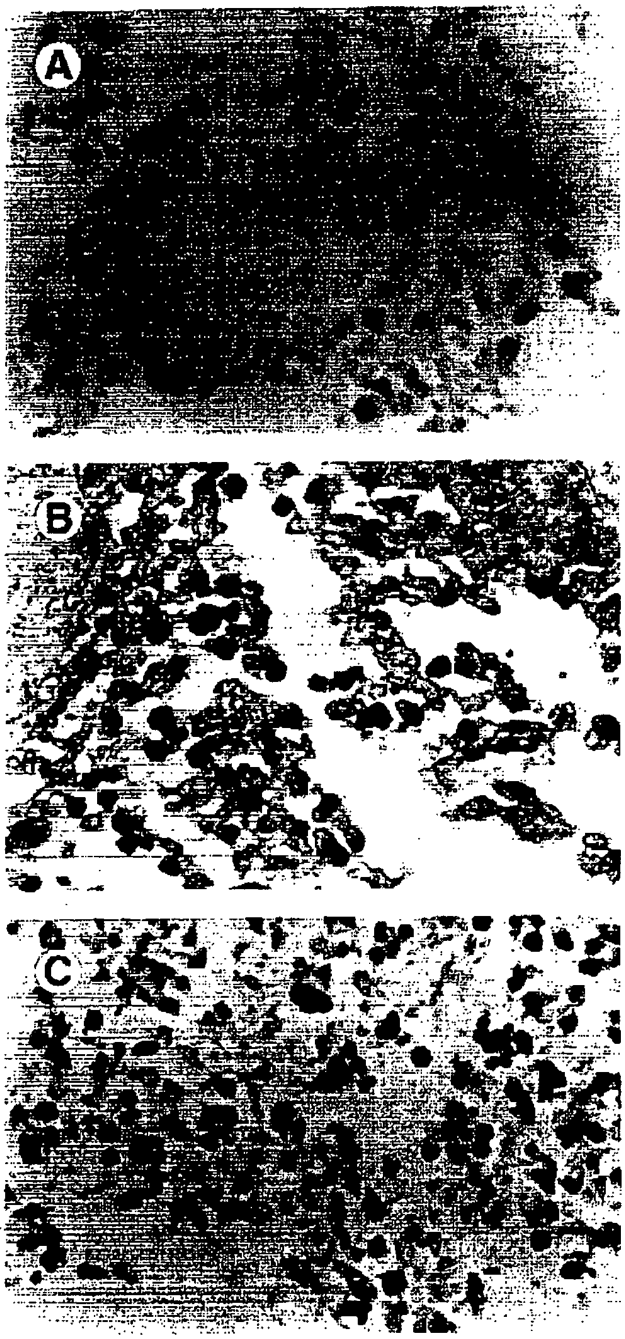

Figure 7. Immunohistochemical analysis of the tumor implanted with MNSM-Alb e/p-TNF-α retroviral–producing cells. Fourteen days after the implantation of the retroviral-producing cells into established subcutaneous HCCs, immunohistochemical analysis was performed to identify the infiltrating cells at the tumor site. The reactive cells were positive to (*A*) Mac-1, (*B*) Lyt2, and (*C*) IL-2R staining (original magnifications: *A–C*, 100×).

cells, however, showed the same growth compared with untransduced parental cells, and both the transduced and untransduced cells were highly resistant to exogenous murine and human recombinant TNF-α (data not shown), indicating that the HCC cell line used in this study was insensitive to TNF-α. TNF-α gene–transduced HCC cells, however, were shown to lose their tumorigenicity when implanted into syngeneic mice. The results shown in this present study are consistent with previous reports that even if parental tumor cells are resistant to TNF-α in vitro, TNF-α gene transduction abrogates their tumorigenicity in vivo.[7,8,42] These results indicate that the antitumor activity of TNF-α is not restricted to cells that are sensitive to TNF-α in vitro, and therefore, TNF-α acts indirectly. Furthermore, we have also shown that the previous inoculation of the TNF-α gene–transduced HCC cells led to acquisition of tumor immunity against parental HCCs in animals.

Asher et al.[8] reported that sarcoma cells modified to express the TNF-α gene inhibited the tumorigenicity of the parental cells when the two were mixed at a ratio of 10:1 and coinjected subcutaneously into syngeneic mice. Han et al.[42] also reported that adenocarcinoma cells modified to produce TNF-α inhibit the tumorigenicity of the parental cells when mixed at a ratio of 1:1 and implanted subcutaneously into athymic nude mice. We show in the present study that HCC cells modified to secrete biologically active TNF-α significantly inhibited the tumorigenicity of the parental HCC cells even when the same number of modified cells was implanted 3 days later into the vicinity of the previous implantation of parental cells. These results indicate that TNF-α–producing tumor cells may function in a paracrine manner, resulting in the inhibition of the growth of unmodified parental tumor cells implanted in the same vicinity. These results also indicate that transfer of the TNF-α gene into tumor cells ex vivo with subsequent reimplantation into the tumor site may be efficacious in suppressing the tumor growth.

However, ex vivo gene therapy requires a surgical procedure to obtain the target tumor cells for gene transfer. Therefore, it is not practical for the treatment of HCC, because frequently HCC is accompanied by liver cirrhosis,[43] with the majority of patients with HCCs unable to withstand invasive surgery because of limited hepatic reserve. In vivo gene therapy is much more applicable for the clinical treatment of patients with HCCs. In the present study, we show that intratumoral administration of retroviruses carrying the TNF-α gene could induce the regression of established HCCs. Most importantly, intratumoral implantation of the cells that produce retroviruses carrying the TNF-α gene completely abrogated established HCCs. Notably, no obvious signs of treatment-related side effects, such as body weight loss, were observed. These results indicate the potential effi-

cacy of gene therapy against HCC by means of retroviral-mediated in vivo transduction of the TNF-α gene.

To achieve a successful in vivo gene therapy, it is extremely important to induce a strong expression of the exogenous genes used for a therapeutic purpose. It was reported already that high levels of TNF-α released by genetically modified tumor cells led to the rapid disappearance of incipient tumors.[2,8] The present study shows that HCC cells infected with MNSM-Alb e/p-TNF-α retroviruses could express the transduced TNF-α gene significantly more strongly than those infected with MNSM-SV 40-TNF-α retroviruses in vitro. In the subsequent in vivo study, it was shown that intratumoral administration of MNSM-Alb e/p-TNF-α retroviruses could induce significantly stronger antitumor effects against established parental HCCs than those of MNSM-SV 40- TNF-α retroviruses and could significantly prolong the survival period of the mice bearing an established HCC. Also, it was shown that intratumoral implantation of the cells that produce MNSM-Alb e/p-TNF-α retroviruses, but not MNSM-SV 40-TNF-α retroviruses, could completely abrogate the established parental HCCs. Furthermore, intratumoral expression of the TNF-α gene was more strongly induced by implantation of MNSM-Alb e/p-TNF-α retroviral–producing cells compared with MNSM-SV 40-TNF-α retroviral–producing ones. These results suggest that increased local concentrations of TNF-α are responsible for tumor regression. In many investigations on gene therapy, viral-derived promoters including the SV 40 early region promoter have been used because they are generally strong and not tissue specific. The results of the present study, however, indicate that the choice of promoters to direct the therapeutic gene is extremely important for effective gene therapy.

Although regression of tumors induced by in vivo transduction of the TNF-α gene appears to be dependent on TNF-α production in the tumor sites, the mechanism mediating the tumor elimination remains unknown. Blankenstein et al.[7,44] reported that when murine plasmacytoma cells were modified to secrete TNF-α and implanted in syngeneic mice, the suppression of tumor growth was linked to macrophage-mediated processes and probably induced by a T cell–independent mechanism. Han et al.[42] showed that human adenocarcinoma cells modified to secrete TNF-α lost their tumorigenicity even when implanted in athymic nude mice that do not have an intact T-cell system. In contrast, Asher et al.[8] reported that when murine sarcoma cells were modified to secrete TNF-α and implanted in syngeneic hosts, tumor growth suppression was linked to T-cell processes. In the present study, we show that when cells that produce the TNF-α gene–carrying retroviruses were implanted into established tumors, massive infiltration of the cells that contained Mac-1, Lyt2, and IL-2R–positive ones, but not L3T4-positive ones, was observed. Our results, therefore, indicate that both the macrophages and the Lyt2-positive T lymphocytes may play important roles in TNF-α–mediated tumor regression.

To prove the usefulness of the strategy using retroviral-mediated in vivo transduction of the TNF-α gene under the transcriptional control of the albumin gene promoter, more investigations have to be performed. Assessment using different HCC cell lines, including human ones, also have to be completed. Furthermore, the value of this strategy has to be estimated in animal models that bear HCCs present in the liver. The results shown, however, indicate the potential efficacy of transferring the TNF-α gene via retroviral vectors in vivo for the treatment of patients with HCCs.

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